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Effects of various Toxeat® concentrations on growth performance, immune response, cecal microflora, and gut morphology in broilers fed with aflatoxin contaminated diets

Soheila SARRAMI[1], Parvaneh JAFARI[1,*], Maryam TAJABADI EBRAHIMI[2], Seyed Naser MOUSAVI[3], Seyed Abdollah HOSSEINI[4]

[1]Department of Microbiology, Faculty of Science, Islamic Azad University, Arak Branch, Arak, Iran
[2]Department of Biology, Faculty of Science, Islamic Azad University, Central Tehran Branch,Tehran, Iran
[3]Department of Animal Science, Islamic Azad University, Varamin-Pishva Branch,Varamin, Iran
[4]Department of Animal Nutrition and Physiology, Animal Science Research Institute,
Agricultural Research Education and Extension Organization, Karaj, Iran

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Abstract: This study was conducted to investigate the effects of Toxeat®, a mycotoxin binder, on functional indices, immune response, intestinal microflora, and gut morphology in broiler chickens fed aflatoxin-contaminated diets. Three hundred Cobb 500 broilers were randomly divided into 6 treatments with 5 replicates, each with 10 birds. The treatments included T_1: negative control (NC), T_2: T_1 + 1 g/ton aflatoxin (positive control: PC), T_3: PC + 1 kg/ton Toxeat®, T_4: PC + 2 kg/ton Toxeat®, T_5: PC + 3 kg/ton Toxeat®, T_6: PC + 1 kg/ton Toxeat® without hydrated sodium calcium aluminosilicate. Growth performance, immune system response, and lactic acid bacteria (LAB) count decreased in the PC group ($P < 0.05$). Surprisingly, humoral and cellular immune response significantly increased in the Toxeat® treatments ($P < 0.05$). Besides, administration of Toxeat® significantly increased weight gain and LAB count compared with the PC group at the end of experiment ($P < 0.05$), whereas intestinal morphology showed no significant changes ($P > 0.05$). Finally, Toxeat® administration (1–2 kg/ton) resulted in the best growth performance and immunity.

Key words: Aflatoxin, Toxeat®, broiler, immune system response, bactic acid bacteria

1. Introduction

Aflatoxins are fungal metabolites that contaminate a wide range of foods and crops. Aflatoxin B1 is the most common form that causes liver damage and cancer in animals [1]. Aflatoxin's toxicity results in weight loss and deficiency of the immune system in birds such as suppression of phagocytic activity and reduced secretion of interferons and immunoglobulins [2]. Aflatoxins reduce lactic acid bacteria (LAB) count in the intestines [3,4]. So far, several physical, chemical, and biological methods have been developed for detoxification of mycotoxins. It was proved that inorganic absorbents such as aluminosilicates could nonselectively bind to toxins [5,6]. However, bioabsorption, biotransformation, and biodegradation are new detoxification methods which rely on structure and enzymes of microorganism. Cell wall components of microorganisms bind to aflatoxins and absorb it. Some microorganisms enzymatically degrade aflatoxins into less toxic or nontoxic metabolite too [7].

Aflatoxin contamination in livestock feed causes economic losses and also the risk of its entry into the human food chain. Thus, we developed a new toxin binder product, Toxeat®, that includes selected LAB, yeast cell walls, and hydrated sodium calcium aluminosilicate (HSCAs) to overcome this severe problem. Efficacy of Toxeat® at three different concentrations was assayed by determining its effects on growth performance, immune system, intestinal morphology, and microbial flora of chickens fed with aflatoxin-contaminated diets.

2. Materials and methods

2.1. Aflatoxin production

For Aflatoxin production, *Aspergillus flavus* (PTCC 5004) was purchased from the Iranian Research Organization for Science and Technology to inoculate rice. Rice was incubated for three weeks at 28 °C. Contaminated rice was powdered and aflatoxin concentrations were assayed using the HPLC method (Waters Alliance 2695 equipped with 2475 fluorescence detector, USA) [1]. Aflatoxin concentrations were 0.4452 ppm (0.2894 ppm of B_1, 0.0222 ppm of B_2, 0.1256 ppm of G1, and 0.008 ppm of G_2). Rice

* Correspondence: p-jafari@iau-arak.ac.ir

powder was mixed with feed ingredients to prepare B1 (1 mg/kg) in the final feed. In this experiment Aflatoxin (1 mg/kg) in the final feed was used (50 times greater than the maximum permissible limit for aflatoxin) (0.02 ppm) [8].

2.2. Mycotoxin binder compounds

Toxeat® is a commercial mycotoxin binder produced by the Tak Gene Zist Co. which contains four strains of *Lactobacillus* spp. and two strains of *Bacillus* spp. (1×10^7 CFU/g of each). The strains were selected based on previous in vitro study and had the highest aflatoxin absorption capacity among the 200 Iranian strains which were isolated from dairy products. Besides these bacterial strains, *Saccharomyces cerevisiae* cell walls and HSCAs that exist in this product can nonselectively absorb all kinds of toxins.

2.3. Experimental groups

For this experiment, three hundred 7-day broiler chickens (*Gallus gallus*), genetic strain Cobb500, were randomly divided into six treatments with five replicates (10 birds per replicate, half of them male and the other half female). The experimental groups included T1: negative control (NC) that received the basic diet with no additive, T2: positive control (PC) that received the basic diet + aflatoxin (1 g/ton), T3: PC + Toxeat® (1 kg/ton), T4: PC + Toxeat® (2 kg/ton), T5: PC + Toxeat® (3 kg/ton), and T6: PC + Toxeat® (1 kg/ton) without the inorganic carrier (HSCAs).

The chemical composition of the feeds used in the experimental diet according to the manuals of cobb500 broiler chickens. During the experiment, soybean and corn were used for formulating the experimental mash diets that were analyzed for DM, CP, and amino acid contents by using near-infrared reflectance at Paya Amin Mehr Laboratory. The metabolizable energy contents of the feed were analyzed by using the regression models introduced by the National Research Council (Table 1).The broilers were vaccinated against bronchitis, Newcastle, and Gumboro diseases in a drug-free program. During the experiment, the temperature and lighting control systems were set according to the Cobb500 Broiler Management Guide. The environmental conditions were the same for all the experimental groups, and the birds had ad libitum access to feed and water. At all stages of the trial, all ethical considerations were followed.

2. 4. Performance evaluation

To compare performance indices; weight gain, feed intake, feed conversion ratio, and survival rate were recorded during the experiments. Body weight and feed intake were recorded, and feed conversion ratio (FCR) was calculated through dividing total feed intake by body weight.

2.5. Immune system assessment

For the contact sensitivity test, five birds from each treatment were challenged with 0.2 mL of 1% Dinitrochlorobenzene (DNCB), (Sigma Aldrich, Germany) in a featherless area on the right side of the body. The thickness of the skin was measured after 24 and 48 h [9]. For evaluation of cutaneous basophil hypersensitivity (CBH), 0.1 mL of Phytohemagglutinin-P (PHA-P), (Sigma Aldrich, Germany) solution was injected in the right foot of birds and variation in toe-web response was calculated after 24 and 48 h [10].

On day 42, blood samples were taken from the broilers' wing veins, and the smears were prepared and fixed in ethanol (Merck, Germany). The percentage of heterophils and lymphocytes were counted to determine the heterophil/lymphocyte ratio [11].

Table 1. Ingredients and composition of basic rations (as nutrition based on %).

Feed ingredient	1–14 (d)	15–28 (d)	29–42 (d)	Nutrition composition	1–14 (d)	15–28 (d)	29–42 (d)
Corn	55	46.08	45	AME (kcal/kg)	2905	2987	3121
Soybean meal	39	29	32.6	Dig lysine	1.156	0.923	0.994
Vegetable oil	1	1.05	3.8	Methionine& Dig cystine	0.834	0.698	0.717
Wheat	0	20	15	Crude protein	22.58	19.25	20.23
Oysters shell	1.3	1.17	1	Calcium	1.068	0.87	0.812
Salt	0.2	0.2	0.1	Phosphorus	0.546	0.42	0.424
*Premixing nutrients	3.5	2.5	2.5	Sodium	0.212	0.187	0.145

* Premix provided the following nutrients in 1 kg of diet: vitamin A, 10000 IU; vitamin D3,3500 IU; vitamin E, 40 IU; vitamin K3, 2 mg; vitamin B1, 2 mg; vitamin B2, 5 mg; vitamin B3, 35 mg; vitamin B5, 13 mg; vitamin B6, 1.5 mg; vitamin B12, 0.01mg; vitamin B9, 1.6 mg; Biotin, 1.5 mg; I, 1.25 mg; Cu, 16 mg; Zn, 100 mg; Se, 0.3 mg; Mn, 120 mg; Fe, 40mg; Choline chloride,350mg; Betaine,150 mg; The level of other nutrient in each kg of base mix: ME (kcal/kg) 2837; CP, 125g; TSAA, 63 g, Dig Lys g, 18; Dig Thr 8.5 g Ca, 218.8 g, Na 24.5 g, AP, 115 g. d: days, Dig: digestible.

Evaluation of hemorrhagic immunity was performed by injection of 0.5 mL of 5% sheep red blood cell (SRBC) on days 21 and 28. Seven days after the injections, blood sample was collected form wing vein. The microtiter hemagglutination method was used to determine the overall response to SRBC, IgG, and IgM concentrations in the serum [12].

2.6. Intestinal morphology

On day 42, three chicks from each treatment were euthanized and their jejunum and ileum sections were washed with 9% saline solution. After the tissues were fixed in 10% buffered formalin (Sigma Aldrich, Germany) and dehydrated, they were embedded in paraffin. Consecutive 5-µm thick sections were cut from the tissues, stained with hematoxylin and eosin (Merck, Germany), and villus length, villus width, and crypt depth were measured by Nikon E100 microscope [13].

2.7. Cecum microbial flora

To determine cecal microflora, 1 g of cecum content in each bird was transferred to a sterile falcon tube on ice and immediately transferred to the laboratory. Fecal specimens were serially diluted in phosphate buffer solution (pH 7.2). For enumeration of LAB, serially diluted samples were cultured on MRS agar medium (Sigma Aldrich, Germany) and incubated at 37 °C for 48 h in a 5% CO_2 atmospheric. EMB Agar medium (Merck, Germany) was used for differentiation and enumeration of *Escherichia coli*, and the plates were incubated at 37 °C for 24 h [14,15].

2.8. Statistical analysis

The results of the experiment were analyzed using a completely randomized design. SAS Institute 9.2 software (2009) was used for the statistical analysis of all data and Duncan's multidomain test at the significance level of 0.05 for the comparison of the means.

3. Results

Our results showed that the administration of aflatoxin significantly reduced weight in the PC group compared to the negative control ($P < 0.05$). Surprisingly, use of Toxeat® inhibited weight loss in the treatment groups ($P > 0.05$).

As was expected, aflatoxin reduced feed intake and therefore increased the feed conversion ratio (FCR) in the PC group (2.38) compared to the NC group (2.02). The Toxeat® supplement significantly improved FCR in treated groups, so there were no significant differences between the Toxeat® treated groups and the NC group with respect to FCR ($P > 0.05$, Table 2).

Skin sensitivity test with DNCB on day thirty-five showed the PC group with diameter of 0.5 mm had the weakest skin responses among all the groups ($P < 0.05$, Table 3). This means that aflatoxin caused significantly decreased cell mediated immune response (Table 3). However, as was expected from the presence of probiotic bacteria in Toxeat®, the immune system was ameliorated in Toxeat®-treated groups ($P < 0.05$, Table 3). In the CBH test, the PC group exhibited the weakest and the T_3, T_5, and T_6 groups the strongest response ($P < 0.05$, Table 3). As obviously seen in Table 3, the percentage of lymphocyte declined in the PC group due to the absorption of aflatoxin. Toxeat® controlled this reduction and ameliorated the side effects of aflatoxin. There were significant increases in the number of immune cells in the Toxeat®-treated groups compared with the PC group ($P < 0.05$, Table 3).

As it can be seen in Table 4, the highest concentrations of the antibodies against SRBC on day twenty-eight were those of the NC, T_3, and T_5 groups with no significant differences between them ($P > 0.05$). However, the highest levels of IgG were found in the T_3 and T_5 groups that received Toxeat® ($P < 0.05$). The highest antibody response to SRBC at the age of thirty-five days amongst the Toxeat®-treated groups belong to T_4 and T_6 ($P < 0.05$) and the highest IgG level to the NC and T_3 groups ($P < 0.05$). However, the IgM results did not show a significant difference in the two periods ($P > 0.05$). In both experimental periods, the lowest antibody titer was against SRBC, and the lowest IgG titer was measured in the PC group ($P < 0.05$).

The length of the villus in both sections decreased in the PC group compared to the other groups numerically ($P > 0.05$). There was no significant difference between T_3 and NC group for villi length to crypt depth ratio (V: C) numerically ($P > 0.05$, Table 5).

As shown in Table 4, aflatoxin reduced the LAB count in the PC group whereas the number of *E. coli* increased simultaneously ($P < 0.05$). Toxeat® at all levels significantly increased the LAB count and decreased the number of *E. coli*. This amelioration of intestinal profile could be the reason for better food absorption and higher FCRs in these groups ($P < 0.05$).

4. Discussion

According to the results of the study, the deleterious effect that aflatoxin has on the feed intake, BW, and FCR in the PC group can be attributed to the lethargy (a feeling of listlessness and general inactivity), anorexia (a loss of appetite), inhibition of protein synthesis, and lipogenesis [16]. Administration of Toxeat®, however, not only improved FCR but also ameliorated the adverse effects of aflatoxin [11,16]. Salem et al. [17] used a commercial biological mycotoxin binder in diets infected with aflatoxin. The commercial product controlled the adverse effects that aflatoxin have on BW and FCR and prevented the incidence of aflatoxicosis in poultries. Similar results were obtained when using Toxeat®, where the effects of probiotics bacteria and HSCAs available in Toxeat® managed to control and/or alleviate the adverse effects that aflatoxin have on BW and FCR.

Table 2. The effect of different doses of Toxeat® on the growth performance of poultry.

Trt	Weight gain (g/bird)		Feed intake(g/bird)	FCR	Livability %
	7 (d)	42 (d)	1–42 (d)	42 (d)	42 (d)
NC	148.1 ± 7.2	2028.9^{a} ± 52.0	4111.4^{a} ± 51.8	2.02^{b} ± 0.03	98^{a} ± 4.4
PC	145.1 ± 8.4	1268.9^{c} ± 184.9	2989.6^{c} ± 237.7	2.38^{a} ± 0.27	79.30^{ab} ± 15.1
T_3	147.4 ± 6.3	1712.8^{b} ± 48.9	3121.3^{c} ± 86.2	1.88^{b} ± 0.01	93.33^{a} ± 8.1
T_4	145.1 ± 3.1	1665.6^{b} ± 98.6	3146.7^{c} ± 146.6	1.89^{b} ± 0.02	94^{a} ± 8.9
T_5	141.6 ± 5.7	1673.4^{b} ± 33.7	3202.6^{bc} ± 63.4	1.91^{b} ± 0.02	80^{ab} ± 18.7
T_6	148.3 ± 4.6	1931.0^{a} ± 94.8	3415.1^{b} ± 191.9	1.87^{b} ± 0.09	66^{b} ± 16.7
P-value	0.52	0.0001	0.0001	0.0001	0.0081
SEM	1.1	49.3	75.8	0.04	3.07

d: day, trt: treatment, $^{a–c}$ Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Table 3. The effect of different doses of Toxeat® on DNCB and CBH at the age of 35 days and percentage of blood cell in broiler chickens at the age of 42 days.

Trt	DNCB (mm)		CBH (mm)		Percentage of blood cell		
	24(h)	48(h)	24(h)	48(h)	H%	L%	H: L
NC	1.39 ± 0.1	0.91^{a} ± 0.1	1.19^{ab} ± 0.5	0.37 ± 0.1	4.40^{d} ± 0.5	95.60^{a} ± 0.5	0.05^{d} ± 0.006
PC	1.58 ± 0.2	0.50^{b} ± 0.07	0.62^{b} ± 0.2	0.22 ± 0.05	8.60^{a} ± 0.5	91.04^{d} ± 0.5	0. 09^{a} ± 0.006
T_3	1.52 ± 0.1	0.91^{a} ± 0.1	1.47^{a} ± 0.3	0.36 ± 0.1	7.20^{b} ± 0.8	92.80^{c} ± 0.8	0.08^{b} ± 0.009
T_4	1.63 ± 0.2	0.80^{a} ± 0.1	0.99^{ab} ± 0.2	0.36 ± 0.1	6.60^{bc} ± 0.8	93.40^{bc} ± 0.8	0.07^{bc} ± 0.01
T_5	1.46 ± 0.2	0.80^{a} ± 0.2	1.34^{a} ± 0.4	0.38 ± 0.08	5.80^{c} ± 0.4	94.20^{b} ± 1.3	0.06^{c} ± 0.005
T_6	1.46 ± 0.2	0.88^{a} ± 0.2	1.29^{a} ± 0.6	0.30 ± 0.009	7.00^{bc} ± 1.5	93.00^{bc} ± 1.5	0.07^{bc} ± 0.01
P-value	0.65	0.01	0.05	0.26	0.0001	0.0001	0.0001
SEM	0.04	0.04	0.09	0.02	0.28	0.28	0.003

DNCB: Dinitrochlorobenzene, CBH: Cutaneous basophil hypersensitivity test, h: hour, H, Hetrophils; L, Lymphocytes; H: L, hetrophil: Lymphocyte, Trt: treatment, $^{a–d}$ Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Decreased IgA and IgG levels in broiler chickens infected with aflatoxin has proven to weaken the immune system [12,18]. The number of heterophils and lymphocytes are increased and decreased in response to aflatoxin toxicity, respectively, causing the lower efficiency of the immune system [16]. Probiotics have many benefits to the host mainly through improving and/or strengthening the immune system [16]. They improve the efficiency of intestinal absorption of food by absorbing aflatoxin from the diet [19]. As the results of the present study indicated, Toxeat® can alleviate suppressive and stressful effects that aflatoxin has on chickens. There is a correlation between the results of our research and the findings of previous studies on immune system response and growth performance. As the literature suggests, the antibody titer is increased by applying *Saccharomyces cerevisiae* in diets infected with aflatoxin [20]. Toxeat® contains yeast wall of *S. cerevisiae*, leading the antibody titer to be increased in groups received Toxeat®. And this finding is consistent with the results of the study mentioned above.

Increased number of gram-negative bacteria and a decrease in population of intestinal LAB are the adverse effects of aflatoxin in chickens [3,4]. The results showed a significant decrease in LAB count, whereas a significant increase in LAB count was observed in Toxeat®-treated groups. LAB can protect poultries against adverse effects

Table 4. The effect of different doses of Toxeat® on the SRBC and immunoglobulin titers (Antiimmunoglobulin titer (Log_2)) and the microbial caecum population (log10 cfu/g) at the age of 42 days.

Trt	28 days			35 days			Cecal microbial population	
	SRBC	IgG	IgM	SRBC	IgG	IgM	*E. coli*	*Lactobacillus*
NC	$4.8^{a} \pm 0.8$	$2.6^{ab} \pm 0.8$	2.2 ± 0.8	$6.2^{a} \pm 0.4$	$4.8^{a} \pm 0.4$	1.4 ± 0.5	$5.5^{b} \pm 0.5$	$8.80^{b} \pm 0.8$
PC	$3.2^{b} \pm 0.8$	$2.2^{b} \pm 0.8$	1.0 ± 0.8	$4.2^{c} \pm 0.8$	$3.4^{c} \pm 0.5$	0.8 ± 0.8	$7.2^{a} \pm 0.5$	$6.83^{c} \pm 0.5$
T_3	$4.6^{a} \pm 0.5$	$3.4^{a} \pm 0.5$	1.2 ± 0.5	$5.0^{bc} \pm 0.7$	$4.2^{ab} \pm 0.4$	0.8 ± 0.8	$4.6^{c} \pm 0.7$	$10.54^{a} \pm 1$
T_4	$4.2^{ab} \pm 0.8$	$2.8^{ab} \pm 0.4$	1.4 ± 1.1	$5.4^{ab} \pm 0.5$	$3.4^{c} \pm 0.5$	2.0 ± 1	$4.6^{c} \pm 0.8$	$10.28^{a} \pm 0.6$
T_5	$4.6^{a} \pm 0.5$	$3.4^{a} \pm 0.8$	1.2 ± 0.8	$4.8^{bc} \pm 0.4$	$3.6^{bc} \pm 0.5$	1.2 ± 0.4	$3.9^{c} \pm 0.6$	$10.35^{a} \pm 0.6$
T_6	$4.0^{ab} \pm 0.7$	$3.0^{ab} \pm 0.7$	1.0 ± 0.7	$5.4^{ab} \pm 0.8$	$3.6^{bc} \pm 0.5$	1.8 ± 1.3	$4.1^{c} \pm 0.3$	$10.61^{a} \pm 0.6$
P value	0.02	0.03	0.37	0.0027	0.0012	0.19	0.0001	0.0001
SEM	0.16	0.13	0.18	0.16	0.13	0.17	0.23	0.28

SRBC: Sheep red blood cell, IgG: Immunoglobulin-G, IgM: Immunoglobulin-M, Trt: treatment, $^{a-c}$ Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Table 5. The effects of Toxeat® on intestinal morphology at the age of 42 days.

Trt Items	NC	PC	T_3	T_4	T_5	T_6	P-value	SEM
Jejunum (μm)								
Vh.	1253 ± 132	1160 ± 78	1376 ± 15	1273 ± 124	1283 ± 118	1290 ± 140	0.3	26.7
Vw.	116.6 ± 5	126.6 ± 11	120 ± 10	123.3 ± 15	123.3 ± 5	116.6 ± 5	0.7	2.1
Cd.	82.1 ± 7.9	84.3 ± 8	87.1 ± 6.6	94.7 ± 5	86.9 ± 14.2	83.0 ± 5.1	0.5	1.9
V: C	15.2 ± 0.3	13.8 ± 1.2	15.8 ± 1.1	13.4 ± 1.7	14.9 ± 2.3	15.5 ± 1.8	0.4	0.3
Ileum (μm)								
Vh.	860 ± 45	816.6 ± 46	826.6 ± 153	813.3 ± 124	823.3 ± 118	830 ± 140	0.1	22.7
Vw.	800 ± 90	836.6 ± 70	896.6 ± 15	813.3 ± 89	803.3 ± 118	810 ± 140	0.8	20.6
Cd.	83 ± 5.1	84.2 ± 13.9	76.3 ± 2.3	84.6 ± 22.2	83.9 ± 7.7	83.3 ± 9.8	0.9	2.4
V: C	10.4 ± 1.1	9.8 ± 1.6	10.8 ± 2.3	10.1 ± 3.7	9.8 ± 1.1	9.9 ± 1.0	0.1	0.4

Vh: Villus height, Vw: Villus width, Cd: Crypt depth, V: C: Villus Height: Crypt depth.

of aflatoxicosis [19]. LAB-containing Toxeat® increased LAB levels in the cecum. It also can counteract the adverse effects of aflatoxicosis in poultry flocks, because of high-potential LAB it contains. Using probiotics in diets infected with the use of probiotic in contaminated diets of aflatoxin not only absorbs poisons from the poultry diet but also, by being present in the intestines of the animal, competes with pathogenic bacteria and alters the microflora of the intestine towards beneficial bacteria, improves the growth function and metabolism [19].

Based on the above mentioned material, in groups receiving Toxeat®, the increase in the number of LAB resulted in improved performance in these groups.

As the literature suggests, lactobacillus bacteria with *S. cerevisiae*, *Lactobacillus plantarum*, bentonite, or zeolite in chicken exposed to aflatoxin-contaminated rations can absorb poison, improve the immune system, increase growth performance and intestinal absorption of food, and reduce the damage of aflatoxin to tissues such as the kidney and liver against the effects of solo aflatoxin in these studies [21–24].

Toxeat® contains yeast, probiotic bacteria, and HSCAs, and the results of the present study—in accordance with the findings of previous studies—confirms its efficiency in controlling and/or alleviating the adverse effects that aflatoxin has on poultries.

Overgrowth of pathogenic bacteria due to aflatoxin intake causes intestinal dysbiosis and results in abnormality

in the physiology of intestinal epithelium. Aflatoxins cause significant reduction in villus length and disrupt feed absorption [25]. In this experiment, villus length and the number of intestinal *E. coli* increased as a consequence of toxin intake. Control of intestinal homeostasis can be an important way to improve the health of the host [25]. This improvement in health was achieved by adding Toxeat® to the contaminated feed in the diet.

Our results showed that Toxeat® was able to improve the immune system response. Furthermore, a reduction in the pathogenic bacteria and an increase in the LAB improved the immune system and growth performance in the groups that received Toxeat®. Toxeat® is a dual-purpose product: it has mycotoxin-binding properties and is also a proper probiotic product for broiler. Therefore, its administration improves growth performance.

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